

treatment of b-Rq mice was associated with elevated serum insulin and decreased blood glucose levels, increased pancreatic insulin content, increased beta-cell mass and rate of beta-cell proliferation, and elevated expression of several genes important for the maintenance of beta-cell function and mass, including IRS-2 and the transcription factors Pdx1, MafA, NeuroD1 and Ngn3. Chronic activation of beta-cell Gq signalling also protected b-Rq mice against hyperglycemia and glucose intolerance induced by consumption of a high-fat diet or treatment with low doses of streptozotocin. Studies with b-Rq-mice lacking IRS2 strongly suggested that IRS2 plays a central role in mediating the beneficial metabolic effects resulting from prolonged activation of beta-cell Gq signalling. In vitro studies demonstrated that the enhanced expression of IRS-2 triggered by activation of beta-cell Gq signaling required PKC-dependent ERK activation. These results suggest that agents aimed at enhancing Gq signaling in pancreatic beta-cells could become clinically useful as antidiabetic drugs.

NIEHS

Chan, Kin

Postdoctoral Fellow

Carcinogenesis

A reporter system for identifying mutagens acting preferentially on single-strand DNA

For most carcinogens, the underlying mechanisms of action are poorly understood. Our recent results show that very strong mutagenesis can be specific to single-strand DNA, and thus can be overlooked by conventional mutagenesis assays. Given the common occurrence of ssDNA in cells, we sought to develop a reporter system in yeast that readily identifies carcinogens that are strongly mutagenic specifically to ssDNA. The reporter genes ADE2, URA3, and CAN1 were inserted into the left subtelomeric region of chromosome V, in *cdc13-1* temperature-sensitive yeast. Shifting to 37°C causes telomere uncapping followed by 5' to 3' resection, exposing a long 3' ssDNA overhang containing the reporter. We induced DNA damage by expressing human APOBEC3G or by acute treatment with sodium bisulfite. These agents were not known to be strong mutagens. However, since they deaminate cytosine in ssDNA, multiple uracils are formed in the ssDNA overhang. Upon return to permissive temperature, the complementary strand of subtelomeric DNA is resynthesized. The presence of lesions in the ssDNA overhang, i.e. abasic sites from excision of uracil or the 6-sulfonyluracil intermediate of the bisulfite reaction, forces the use of error-prone translesion DNA synthesis (TLS), creating a distinctive strand-coordinated, multi-mutation signature. Treatment with either agent caused a >1,000-fold increase in simultaneous loss of CAN1 and ADE2 function. In contrast, neither agent induced mutations in controls where the reporter was maintained in a dsDNA state. Sequencing of 114 multiply-mutated reporter isolates revealed 541 mutations, of which >97% originated from cytosines in the ssDNA overhang. Deletion of the uracil-DNA N-glycosylase gene UNG1 resulted in a 5-fold increase in APOBEC3G-induced mutagenesis, and all mutations were C to T transitions, in agreement with Ung1-catalyzed removal of uracil from DNA. In contrast there was no effect on bisulfite mutagenesis, consistent with Ung1 being inefficient at excising 6-sulfonyluracil. Finally, Pol zeta is the main TLS polymerase for mutagenic bypass, since deletion of REV3 decreased the mutation frequency by 90%. Altogether, our results show the strong in vivo ssDNA mutagenic activity of a ubiquitous physiological factor (APOBEC3G) and a known food contaminant (bisulfite). In turn, this successful application of the reporter system provides validation for our approach to identifying other ssDNA-specific mutagens in a high throughput manner.

NIEHS

Ghosh, Swati

Visiting Fellow

Cell Biology - General

An integrated approach reveals that Tet1 maintains mouse embryonic stem cell identity partly by regulating LIF dependent Stat3-mediated gene activation

Embryonic stem cells (ESCs) maintain an unique epigenetic state that enables both self-renewal and differentiation into all embryonic lineages. Because of their ability to differentiate into any of over 200 cell types in adult body, ESC-based therapies have been proposed for regenerative medicine and tissue replacement after injury or disease. The development of such therapies, however, largely depends on our complete understanding of the genes that maintain the self-renewal and pluripotency properties in ESCs, which define the ESC identity. With the goal of predicting novel regulators of ESC identity, we developed a meta-analytic approach that integrated over 60 previously published microarray gene expression datasets in mouse ESCs and differentiated cells. Our analysis revealed Tet1, an enzyme proposed to promote DNA demethylation, as one of several genes to have a previously unknown role in the maintenance of ESC identity. Using RNAi, we validated that Tet1 indeed is required to main ESC identity as Tet1 knockdown (KD) resulted in the differentiation of ESCs. To determine what role Tet1 plays in regulating transcriptional networks influencing self-renewal and pluripotency, we performed microarray expression profiling in control and Tet1 KD cells to identify genes differentially expressed upon Tet1 KD. Bioinformatics analysis of global gene expression changes upon Tet1 KD versus expression changes observed after KD/knockout of any of over 20 other pluripotency-associated factors (gathered from published reports) revealed that genes that are differentially expressed upon Tet1 KD underwent similar expression changes upon leukemia inhibitory factor (LIF) withdrawal. This led us to predict a possible functional interaction between Tet1 and LIF signaling. LIF signaling, mediated by downstream transcription factor Stat3, is known to promote self-renewal and pluripotency in mouse ESCs. Withdrawal of LIF from the culture media leads of differentiation of mouse ESCs. To explore the possible functional interaction between Tet1 and LIF/Stat3 signaling, we examined the ability of Stat3 to bind to its target sites on chromatin in the absence of Tet1. ChIP experiment using Stat3 antibody revealed the loss of Stat3 binding upon Tet1 KD, which was accompanied by loss of expression of Stat3 target genes. These data support that Tet1 is indispensable for maintaining the ESC identity, and that LIF induced Stat3-mediated gene activation in mouse ESCs is dependent on Tet1.

NIEHS

Clement, Tracy

Postdoctoral Fellow

Cell Biology-Cytoskeleton, Extracellular Matrix, and Structural Biology

Testis Expressed Actin-like 7b (Actl7b) is Required for Mouse Spermatid Morphogenesis and Male Fertility

Human male infertility is often associated with a high incidence of abnormally shaped sperm heads, suggesting that cytoskeletal regulation is important for male fertility. During spermiogenesis round spermatids differentiate into elongated and then condensed spermatids followed by spermiation. The involvement of filamentous actin (F-actin) has been suggested for several aspects of spermatid differentiation, including acrosome formation and attachment to the nucleus, formation of tubulobulbar complexes, cytoplasmic removal, and spermiation. Although the structural components and morphological changes in the complex process of spermiogenesis have been described in detail, relatively little is known about the mechanisms that drive these structural changes. Actin-like 7b (Actl7b) is an orphan actin related protein (ARP) family member. Actl7b is an intronless gene expressed in spermatids and conserved in mammals. Immunohistochemistry and indirect immunofluorescence were utilized to investigate the expression of ACTL7B. It is localized in the cytoplasm of round and elongating spermatids and co-localizes with phalloidin labeled F-actin in or around the forming acrosome,

suggesting a role in this process. To determine the functional relevance of Actl7b, knockout mice were generated from targeted ES cells, obtained from the KnockOut Mouse Project (KOMP) Repository, in which the Actl7b coding region was replaced with a LacZ reporter sequence. X-gal staining of tissues from heterozygous animals revealed that Actl7b is expressed in the testis and, unexpectedly, the brain. While Actl7b knockout mice develop to adulthood and appear normal, breeding studies revealed that Actl7b knockout males mate and produce vaginal plugs, but are infertile. Testis and epididymal weights and sperm counts are lower in knockout males than in wild type males. In addition, sperm heads are misshapen with a rounded appearance. Most of these sperm are immotile, with less than three percent showing minimal flagellar movement. These results indicate that Actl7b is required for spermatid morphogenesis, sperm motility, and male fertility. They also suggest that ACTL7B is either the F-actin recognized by phalloidin or is required for F-actin assembly in spermatids. This research was supported by the Intramural Research Program of the NIH.

NIEHS

Charles, Georgette

Postdoctoral Fellow

Developmental Biology

Regulation of APA site choice in the maintenance of ES cells

More than half of mammalian genes have multiple or “alternative” polyadenylation (APA) sites, primarily within the 3′UTR. APA site choice, either proximal or distal, results in respectively a shorter or longer 3′UTR, which often contains cis elements, negatively impacting mRNA stability or translation. Proximal site preference and shorter 3′UTRs have been observed in embryonic stem cells (ESCs), whereas distal site and longer 3′UTRs have been observed during embryonic development. Control of APA site choice and the biological significance for site preference is not fully understood. We hypothesize that a preference for proximal APA sites in ESCs have implications in their ability to self-renew and differentiate. Using RNAi screens in ESCs, we identified Fip1L1 and Cpsf3 as candidates involved in self-renewal. Fip1L1 and Cpsf3 are known members of the cleavage and polyadenylation specificity factor (CPSF) mRNA 3′ processing complex, which is required for 3′ cleavage and polyadenylation. Fip1L1 and Cpsf3 are down-regulated during ESC differentiation. We discovered that silencing of these factors leads to decreased Oct4 expression and ES cell differentiation, indicating their requirement in self-renewal. Given the prerequisite for CPSF complex to specify the cleavage site, we hypothesized a role for the CPSF complex in the regulation of APA site choice. Global polyadenylation site sequencing and differential 3′UTR expression analyses revealed that Fip1L1 and Cpsf3 depletion results in distal APA site usage and longer 3′UTRs for many genes. Many of these putative CPSF targets are highly expressed in ESCs compared to other cell types. This is consistent with 3′UTR-lengthening during ES cell differentiation and embryonic development, accompanied by reduced gene expression. Our data supports a model that ESC maintenance is regulated by high expression of Fip1L1 and Cpsf3, which specify proximal APA site choice and shorter 3′UTRs in ESCs to maintain mRNA transcript stability. De novo sequence analysis within the proximal and distal APA sites for validated targets revealed putative sites for several miRNAs. Currently, we are exploring the role of these miRNAs in conjunction with longer 3′UTRs in ESC self-renewal and differentiation commitment. Our findings provide answers to how APA site choice is controlled in ES cells and contextualizes the significance of proximal APA site choice in ES cell self-renewal, pluripotency, and in early development.

NIEHS

Hamel, Brant

Postdoctoral Fellow

Endocrinology

The N-terminus of the glucocorticoid receptor regulates its nucleocytoplasmic localization

The glucocorticoid receptor (GR) is a nuclear receptor that binds to the hormone cortisol, released in response to stress, to mediate the activation and repression of downstream genes controlling diverse physiological processes from glucose metabolism to suppression of the immune response. GR is a major target of many steroidal anti-inflammatory drugs in clinical use. Unlike related nuclear receptors, GR is predominantly located in the cytoplasm in the absence of hormone and translocates to the nucleus only upon ligand binding. The controlled localization of GR is critical for its ability to activate target genes in response to stimulation from exogenous or endogenous ligands. Multiple motifs have been discovered that influence the subcellular localization of GR including nuclear localization, export, and retention sequences. However, all of the known motifs are located within or between the DNA and ligand-binding domains, while the N-terminal domain has not been shown to play a role in controlling the localization of GR. Using both confocal microscopy and a novel image-based flow cytometric assay, we analyzed a series of nested deletion mutants within the N-terminal domain of GR and identified a deletion, d277-297, that showed a significant increase in basal nuclear localization in the absence of hormone (nuclear localization similarity score of 1.02 versus 0.09 wild-type, $p < 0.0001$). Sequence conservation analysis using a multiple sequence alignment of GR from 35 species illustrated that the residues 277-97 comprised the most highly conserved region in the N-terminus, supporting an important and conserved function for these residues. Furthermore, secondary structure prediction algorithms predict the formation of a beta-sheet in this area that could be important for mediating protein-protein interactions necessary for proper localization of GR. Targeted mutagenesis of this motif lead to the discovery that when 4 residues, K277, E279, K280, and E281, were mutated to alanine, GR had almost complete nuclear localization in the absence of hormone (nuclear localization similarity score of 2.05 versus 0.09 wild-type, $p < 0.0001$). This study is the first to implicate a specific motif in the N-terminal domain of GR as being critical for its proper localization. The mutants will be used in order to evaluate the physiological significance of altered GR localization.

NIEHS

Joubert, Bonnie

Research Fellow

Epidemiology/Biostatistics - Etiology, Risk, and Prevention

Epigenome-wide association study identifies DNA methylation differences in cord blood related to in utero tobacco smoke exposure

Maternal smoking during pregnancy causes adverse health outcomes in offspring. The 2006 US Surgeon General's report concluded that the evidence is sufficient to infer a causal relationship between in utero tobacco smoke exposure and low birth weight, sudden infant death syndrome, and reduced lung function and suggestive for a causal relationship with preterm delivery and some childhood cancers (leukemias, lymphomas, and brain tumors). However, the mechanisms involved in these relationships are not well understood. Data from mouse models has shown that some in utero exposures cause epigenetic changes in the offspring but data in humans are limited and doses given in mouse models are often substantially higher than human exposures. Evaluating the effects of in utero exposures on DNA methylation in cord blood in humans can help elucidate epigenetic mechanisms relevant to childhood and adult disease. In this study, we evaluated the relationship between tobacco smoke exposure during pregnancy and epigenome-wide DNA methylation in cord blood, using the recently released Illumina Infinium HumanMethylation450 BeadChip. We examined maternal plasma cotinine (an indicator of tobacco smoke exposure) measured at gestational week 18 in relation to DNA methylation at 473,844

CpG sites (CpGs) in 1,062 newborn cord bloods from the Norwegian Mother and Child Cohort Study (MoBa). We observed differential DNA methylation at 26 CpGs mapped to 10 genes at epigenome-wide significance ($p\text{-value} < 1.06 \times 10^{-7}$). We observed replication in 36 US cord blood DNA samples, most notably for three CpGs in genes involved in the detoxification of components of cigarette smoke. Our findings also identified altered methylation status among three of the top genes that represents a novel pathway of fetal response to in utero tobacco smoke exposure. Our study of maternal smoking in relation to epigenetic modifications in infants in the MoBa cohort is the largest and most extensive to date. In addition to the large sample size and the use of a platform with comprehensive genome-wide coverage, our study included assessment of maternal smoking by means of a highly sensitive assay for the nicotine metabolite cotinine, a well-validated biomarker. Our findings suggest epigenetic mechanisms for the effects of in utero exposure to tobacco smoke.

NIEHS

Su, Dan

Postdoctoral Fellow

Epigenetics

Chromatin state primes stress specific p53-regulated gene responses

In genotoxic stress, the tumor suppressor p53 plays a crucial role in cell fate, regulating cell-cycle arrest, senescence, or apoptosis by transactivating its myriad target genes. It is well established that p53 recognizes specific DNA sequences, yet little is known about factors determining its occupancy in vivo on chromatin. Understanding how chromatin states regulate p53 access to DNA and drive the diversity in p53 binding and transactivation may lead to novel targets to harness the apoptotic potential of p53. Here, we mapped p53 binding and dynamic chromatin changes in lymphoblastoid cells by ChIP-seq and studied the relevance of chromatin state to stress-specific p53 occupancy and gene expression. Doxorubicin (Doxo), ionizing radiation, or Nutlin-3 was used to induce p53 through DNA damage or MDM2 inhibition. ChIP-seq analysis detected >3000 p53 binding regions with distinctive patterns between exposures. Untreated cells exhibited p53 binding at a small portion of its binding sites, however, the presence of p53 was associated with higher RNAPol II levels ($p < 0.0001$), open chromatin states (ENCODE H3K27Ac, H3K4me3, etc.), and was predictive of time-dependent gene expression kinetics after Doxo exposure. We examined exposure-induced chromatin status and observed that global H3K4me3 changes were distinctive between exposures and were correlated with gene expression ($r^2 = 0.42$, $p < 0.0001$). H3K4me3 levels within the gene body prior to exposure were positively correlated with gene expression but negatively correlated with fold-change gene expression, with known p53 targets overrepresented among the low expression, high fold-change genes. Comparing ENCODE transcription factor (TF) binding profiles (e.g. POU2F2, TCF12, p300, etc.) with p53 binding sites, these TFs occupy many of the accessible chromatin regions prior to p53 binding (32%), suggesting that clustered TF sites may regulate DNA access for p53. The p53 peaks in TF clusters display significantly higher average sequence conservation with syntenic mouse regions (47% vs 31%, $p < 0.0001$), suggesting the possibility of evolutionary selection for TF binding clusters that include p53. Dynamic changes of chromatin insulator protein CTCF were also analyzed by ChIP-seq/PCR. We observed distinct changes in CTCF at p53 target genes (e.g. GDF15) between exposures. Together these data identify stress-specific dynamic chromatin changes, which underline the epigenetic mechanisms of p53-dependent transactivation.

NIEHS

Roberts, Steven

Postdoctoral Fellow
Genomics

A permanent record of transient hyper-mutation associated with single-strand DNA in human cancers

Frequency, location and timing are key parameters determining biological outcomes of mutations. Recent sequencing of human tumors has enabled us to address how these parameters contribute to a cancer mutator phenotype. While inactivation of DNA repair can lead to persistent, high mutation rates, few such examples are reported in cancers. Transient acquisition of multiple mutations in one or a few cell generations is an alternative and could produce synergistic or compensatory changes that provide growth advantage. We have shown that chronic DNA damage can generate clusters of simultaneous multiple mutations via transient mutagenesis. Genome sequencing of methyl methanesulfonate treated yeast revealed mutation clusters composed of "strand-coordinated" changes of either cytosines or guanines in the same strand, indicating the mutations were induced in the same generation. Mutation patterns and genetic controls suggested these mutations resulted from alkylations in long single-strand (ss)DNA formed at double-strand breaks and replication forks. Here, we report simultaneous clustered mutations in human cancers. We designed a bioinformatics approach to search for mutation clusters in large datasets of somatic mutations. Among 328,040 mutations identified in whole-genome sequencing of 23 multiple myelomas, 2 head-and-neck squamous cell carcinomas, 7 prostate and 9 colorectal cancers, we identified 635 clusters within the 4 cancer types. Similar to our yeast findings, both mutated A:T and G:C base pairs in clusters were highly strand-coordinated, indicating simultaneous occurrence of the mutations. One unusual class of clusters was composed entirely of mutated cytosines or guanines and resided close to chromosome rearrangement breakpoints. Surprisingly, nearly all of these mutations occurred at a motif targeted by APOBEC family cytosine-deaminases. These enzymes specifically modify ssDNA, suggesting that mechanisms similar to those in yeast may contribute to mutation cluster formation in human cancers. Mutation at the same motif was enriched throughout the genomes of all tumors analyzed, accounting for up to 50 percent of total cancer-specific mutations and suggesting that even scattered mutations could occur simultaneously, potentially through APOBEC activity on multiple exposed ssDNA regions. Thus hyper-mutation via multiple simultaneous changes in randomly formed transient ssDNA may be an important mechanism for rapid production of genetic variation in cancer.

NIEHS
Zheng, Xiaofeng
Postdoctoral Fellow
Genomics

Identification of a novel component of the self-renewal circuitry conserved in mouse and human ES cells

Embryonic stem (ES) cells have two unique characteristics: self-renewal and pluripotency. Understanding the molecular basis of these defining features of ES cells will provide insights to mammalian embryonic development and facilitate the use of pluripotent stem cells for disease modeling, drug discovery, and stem cell therapies. To systematically study self-renewal and pluripotency, we previously carried out a genome-wide RNAi screen in mouse ES cells. We identified over 100 genes whose down-regulation caused differentiation, including genes encoding members of the Ccr4-Not protein complex. The Ccr4-Not complex is known for the regulation of transcription and mRNA stability, and has been implicated in various cellular activities such as DNA repair, spindle positioning, microtubule length regulation, and histone methylation. However, its role in self-renewal is not understood. We found that three components of the Ccr4-Not complex, Cnot1, Cnot2, and Cnot3, are important for maintaining mouse ES cell identity. Genes corresponding to these three proteins are highly expressed in ES cells and down regulated during differentiation, and they are also enriched in the inner cell mass of the blastocyst stage embryos. In mouse ES cells, global gene expression analysis

indicated that silencing Cnot1, Cnot2, or Cnot3 induces differentiation predominantly into the trophoctoderm lineage. Cnot3 ChIP-on-chip showed that it occupies a unique set of gene promoter regions. Consistently, gene expression analysis showed that the Cnot genes do not impinge on previously known self-renewal transcription factors or pathways. Instead, genetic epistasis analysis demonstrated that they maintain mouse ES cell self-renewal by repressing the expression of early trophoctoderm transcription factors such as Cdx2. Importantly, we found that Cnot1, Cnot2, and Cnot3 are also required for the maintenance of human ES cells, and silencing them in human cells mainly lead to trophoctoderm and primitive endoderm differentiation. Together, our results indicate that Cnot1, Cnot2, and Cnot3 represent a novel component of the core self-renewal and pluripotency circuitry conserved in mouse and human ES cells, and our approach illustrates the power of RNAi and forward genetics for the systematic study of ES cell biology.

NIEHS

Lowe, Julie

Postdoctoral Fellow

Immunology - Innate and Cell-mediated Host Defenses

An unexpected role for p53 in NF-kappaB-mediated inflammatory responses

The transcription factors p53 and NF-kappaB are key mediators of cellular stress such as genotoxic stress. While NF-kappaB is important in inflammation, little is known about the role of p53 in this process. Here, we show that p53 activation in human primary macrophages by the genotoxins 5-Fluorouracil (5-FU) and Doxorubicin (DXR) or by the p53 stabilizer nutlin-3 leads to expression of several pro-inflammatory genes. This effect is specific to human primary macrophages. Although some pro-inflammatory genes are induced to levels typical of p53 targets such as p21, there is much greater induction of IL-6. IL-6 mRNA is induced to high levels within 1 hr after treatment, followed by accumulation of secreted protein. ATM, an upstream regulator of both p53 and NF-kappaB in the DNA Damage Response (DDR), as well as p53 and NF-kappaB are required for IL-6 induction based on responses to their respective inhibitors KU55933, Pifithrin-alpha and Bay-11-7082. While DXR and 5-FU are known to activate NF-kappaB, we show that NF-kappaB is also activated by nutlin-3, presumably via the DDR since nutlin-3 induces gamma-H2AX foci. In response to nutlin-3, IkappaBalpha is phosphorylated and degraded, and the NF-kappaB subunit p65 is transported to the nucleus. Additionally, we show that nutlin-3 induces p65 phosphorylation at S276, which is required for IL-6 induction. Thus, p53 and NF-kappaB can cooperate to induce IL-6 in primary human macrophages in an ATM-dependent manner. Interestingly, the kinetics and magnitude of IL-6 expression by nutlin-3 mirrors that of TLR-dependent IL-6 expression suggesting an equally important biological response. Microarray analyses of RNA from primary macrophages demonstrate limited similarity in gene expression after brief treatments with nutlin-3 and LPS (2 hr), and most genes that are highly induced by both treatments are pro-inflammatory. Contrary to commonly held views, these results show that the relationship between p53 and NF-kappaB may not always be inhibitory and shed new light on the function of p53 in innate immunity. The positive feedback between p53, NF-kappaB and rapid cytokine production may assure innate immune protection under conditions of genotoxic stress. These findings also have important health implications since many environmental agents can induce p53 and NF-kappaB and a derivative of nutlin-3 along with 5-FU and Doxorubicin are currently used or being tested as chemotherapeutic agents.

NIEHS

de Marchena Powell, Jacqueline

Postdoctoral Fellow

Neuroscience - Cellular and Molecular

A novel approach to isolate the function of the galanergic subpopulation of the locus coeruleus

The neuropeptide galanin (GAL) is released as a co-transmitter by a variety of neuronal cell-types throughout the central and peripheral nervous system, including a reported 80% of noradrenergic neurons of the locus coeruleus (LC). Interestingly, GAL has been implicated in a variety of neurological disorders, including Alzheimer's disease. To better understand where GAL-expressing noradrenergic neurons are located and to which brain areas they project, we are using a dual recombinase-based strategy to specifically label this noradrenergic subpopulation. Using this genetic strategy, we have examined the noradrenergic nuclei of the murine hindbrain and found that GAL is primarily expressed in the LC and that the subpopulation of LC neurons expressing GAL increases significantly between postnatal days (P)7, P28, and P56. We are also determining where these GAL-expressing neurons project throughout the brain, with particular emphasis on the hippocampus, an area of the brain critically important in learning & memory. While global GAL knockout mice show deficits in hippocampal-dependent learning & memory, it is unclear how GAL from the LC specifically contributes to this phenotype. To overcome this limitation we are taking advantage of the unique LC-specific overlap of dopamine β -hydroxylase (Dbh) and Engrailed-1 (En1) gene expression to design an intersectional strategy that selectively knocks out GAL in the LC. To target this population, we have designed a completely novel knockout strategy that makes cre expression contingent upon Dre-mediated recombination. For this strategy, we have generated two new knock-in alleles: (1) En1::Dre and (2) Dbh::Dre dependent cre. Crossing these two alleles results in cre expression that is restricted to LC neurons. When En1::Dre; Dbh::Dre dependent cre mice are then crossed to a conditional Gal allele, GAL will be knocked out exclusively in the LC. This genetic scheme allows us to circumvent secondary effects that come from knocking out GAL in all cell-types. Excitingly, these same mouse lines can also be used to conditionally knock out any floxed allele in the LC. This model will allow us to be the first to discern how the specific loss of GAL in this neuronal subpopulation affects neurodevelopment and learning & memory.

NIEHS

Gu, Zhenglin

Research Fellow

Neuroscience - General

Cholinergic coordination of pre- and postsynaptic activity induces timing-dependent hippocampal synaptic plasticity

Correlated pre- and postsynaptic activity is the core element in inducing Hebbian plasticity, including spike timing-dependent plasticity (STDP). However, little is known about the physiological events that could mediate such correlation. Recently we have found that correlated cholinergic input can induce STDP-like $\alpha 7$ nicotinic acetylcholine receptor (nAChR)-dependent hippocampal synaptic long-term potentiation (LTP) or short-term depression (STD). $\alpha 7$ nAChRs, as well as other neuromodulator receptors, are localized at both pre- and postsynaptic sites of glutamatergic synapses, and thus providing a potential mechanism to coordinate pre- and postsynaptic activities to induce synaptic plasticity. To test this, we directly monitored the pre- and postsynaptic activities of hippocampal CA3 Schaffer collateral (SC) to CA1 synapses with genetically-encoded calcium indicators (green GCaMP3 and red R-GECO1) in septo (providing cholinergic innervation)-hippocampal co-cultures. R-GECO1 (in neuron specific synapsin promoter-driven AAV virus) was microinjected into hippocampal CA1 neurons and the dendritic spines in the CA1 SR (stratum radiatum) layer were monitored as postsynaptic activity; GCaMP3 was microinjected into CA3 neurons and the projecting SC axons in CA1 SR layer were

monitored as presynaptic activity. We found that the alpha7 nAChR-dependent LTP (induced by pairing cholinergic pathway 100 ms before SC pathway) involves prolonged enhancement of both pre- and postsynaptic activities, and both were abolished in alpha7 nAChR knockout slices. Restoring alpha7 nAChRs to either pre- or postsynaptic sites in knockout slices only resulted in short-term potentiation at either site. Restoring alpha7 nAChRs to both sites of knockout slices was required to restore the LTP. On the other hand, the alpha7 nAChR-dependent STD (induced by pairing cholinergic pathway 10 ms before SC pathway) involves transient depression of both pre- and postsynaptic activities, and both were abolished in knockout slices. Restoring alpha7 nAChRs to both sites was also required to restore the STD in knockout slices, while there was no effect on either site if only restored to one site. These results suggest that correlated cholinergic input, through the alpha7 nAChRs, can coordinate pre- and postsynaptic activities to induce synaptic plasticity, providing a novel mechanism for neuromodulators to precisely modulate network activity during higher brain functions.

NIEHS

Smith, Lindsay

Postdoctoral Fellow

Pharmacology and Toxicology/Environmental Health

Glucocorticoid Receptor (GR) Regulation of P-glycoprotein (Pgp) at the Blood-Brain Barrier (BBB)

The blood-brain barrier (BBB), comprised of the brain capillary endothelium, provides a major obstacle to drug delivery to the brain. An important element of barrier function is the ATP-driven drug efflux transporter, Pgp, which exhibits broad substrate specificity and high luminal plasma membrane expression in brain capillaries. Previous studies demonstrated that certain ligand-activated nuclear receptors upregulate ATP-driven transporter expression at the BBB. Here, we examined whether GR, a ligand-activated nuclear receptor targeted by an extensive class of synthetic glucocorticoids (GCs), regulates Pgp at the BBB. The effects of synthetic GCs on BBB properties are poorly understood. However, given the potent anti-inflammatory properties of synthetic glucocorticoids, these drugs are routinely used for the treatment of cerebral edema during brain-tumor chemotherapy. Therefore, it is critical to understand how synthetic GCs modulate BBB properties. We hypothesize that synthetic GCs increase the activity and expression of Pgp, thereby limiting the efficiency of drug delivery to the brain. The basal expression of GR in rat brain capillaries was confirmed by western blotting. Adrenalectomized (depleted of endogenous GCs) and intact rats were injected with the synthetic glucocorticoid dexamethasone and 24 hours later brain capillaries were isolated for Pgp transport activity analysis (confocal imaging of accumulation of fluorescent substrate in the capillary lumen) and Pgp protein expression (western blots). In-vivo dexamethasone treatment of both intact and adrenalectomized rats significantly increased Pgp activity and protein expression in brain capillaries. Exposing capillaries (from adrenalectomized and intact rats) to dexamethasone in vitro increased Pgp activity and protein expression in a concentration-dependent manner. These increases were abolished by co-treatment with the GR antagonist RU486. These results show that dexamethasone treatment, in the presence and absence of endogenous GCs, increases the activity and expression of the potent drug efflux pump, Pgp, effectively tightening the barrier for a large number of therapeutic drugs. Thus, the use of synthetic GCs as adjuvants may hinder delivery of other therapeutic drugs to the brain.

NIEHS

Verhein, Kirsten

Postdoctoral Fellow

Pharmacology and Toxicology/Environmental Health

Differential susceptibility to ozone-induced lung inflammation maps to mouse chromosome 17: role of Notch receptors

Ozone (O₃) is a highly toxic air pollutant and worldwide public health concern. O₃ exacerbates preexisting lung conditions and causes airway hyperresponsiveness and inflammation. Mechanisms of genetic susceptibility to O₃-induced airway inflammation are not completely understood. We identified a significant quantitative trait locus on mouse chromosome 17 (Inf2) that contains candidate susceptibility genes for O₃-induced airway inflammation, including histocompatibility genes and the Tnf (tumor necrosis factor) gene cluster. Also located in Inf2 is Notch4, and proximally adjacent to Inf2 is Notch3. Notch receptors are cell surface receptors important in development and immune cell differentiation. We hypothesized that Notch3 and Notch4 are determinants of susceptibility to O₃-induced airway inflammation. To test this hypothesis, wild type (B6129SF1, WT), Notch3 (Notch3^{-/-}) and Notch4 (Notch4^{-/-}) knockout mice were exposed to O₃ (0.3 ppm) or filtered air continuously for 6-48 hr. Immediately after exposure, airway inflammation and injury was assessed using protein concentration (a marker of lung permeability) and inflammatory cells in bronchoalveolar lavage fluid (BALF). O₃ significantly increased BALF protein in all genotypes, but greater concentrations were found in Notch3^{-/-} compared to WT (24, 48 hr), and concentrations were greater in Notch4^{-/-} mice compared to Notch3^{-/-} (24, 48 hr). Greater mean numbers of BALF neutrophils were found in Notch3^{-/-} and Notch4^{-/-} mice compared to WT (24, 48 hr). Expression of whole lung Tnf was significantly increased after O₃ (24 hr) in all genotypes, and was greater in Notch3^{-/-} and Notch4^{-/-} compared to WT. Pre-treatment with the TNF α inhibitor etanercept significantly attenuated the enhanced O₃-induced BALF neutrophils in Notch3^{-/-} and Notch4^{-/-} relative to WT. O₃-induced transcript expression of other Inf2 genes was not different between genotypes. We then used mRNA transcriptomics analyses to further investigate the role of Notch3/4. Statistical and visual data mining approaches identified differentially expressed genes basally [e.g. Gbp1, Cntn1] and after O₃ [e.g. Ccl7, Il33] between WT and KO mice. Results are consistent with the hypothesis that Notch3 and Notch4 are susceptibility genes for O₃-induced airway inflammation. Furthermore, results suggest an important interaction between Notch3, Notch4, and Tnf. These novel findings suggest Notch receptors protect against the innate immune inflammatory response to O₃.

NIEHS

Wang, Qingshan

Postdoctoral Fellow

Pharmacology and Toxicology/Environmental Health

Substance P exacerbates neurotoxins-induced nigral dopaminergic neurodegeneration through activation of microglial NADPH oxidase

Dysregulation of substance P (SP), a major endogenous neuropeptide present in the striatonigral projecting pathway, has been linked with Parkinson's disease (PD). However, roles of SP in regulating long-term survival of dopaminergic neurons in the substantia nigra (SN) remain unstudied. Here, we demonstrate that SP exacerbates dopaminergic neurodegeneration in rodent PD models through augmenting microglia-mediated neuroinflammation. Two in vivo rodent PD models were employed: 1) a single dose of LPS or 2) repeated MPTP regimen in SP-deficient (TAC1^{-/-}), neurokinin-1 receptor (conventional G-protein-coupled receptor for SP) knockout (NK-1R^{-/-}) or wild type (WT) mice. We found that lack of endogenous SP significantly decreased LPS- or MPTP-induced dopaminergic neurotoxicity in both SN and striatum and brain neuroinflammation. Surprisingly, no difference of the above-mentioned changes were observed between NK-1R^{-/-} and WT mice, suggesting that these synergistic effects of SP and neurotoxins are not mediated through the conventional NK-1R. In midbrain neuron-glia cultures, SP enhanced both LPS- and MPP⁺-induced dopaminergic neurodegeneration and

neuroinflammation with an interesting bimodal dose-response curve: effective in nanomolar (10^{-8} ~ 10^{-7} M) and subpicomolar (10^{-14} ~ 10^{-13} M) concentrations, but not in between. Further studies revealed that subpicomolar SP, which is released from terminals of the striatonigral neurons, diffused out synaptic junction and acted on the surrounding microglia. We identified NADPH oxidase, an important superoxide-producing enzyme in microglia, was a novel NK1R-independent target responsible for the synergistic effects of SP and neurotoxins. Additional mechanistic studies showed that SP directly bound to gp91phox, the membrane subunit of NADPH oxidase, through C-terminal PHE-GLY-LEU-MET domain and then either alone or with LPS induced membrane translocation of cytosolic subunits p47phox and p67phox, resulting in NADPH oxidase activation. Signaling studies further showed that SP was capable of enhancing production of TNF α by potentiating LPS-induced activation of MAPK and NF- κ B pathway through NADPH oxidase-dependent manner. Altogether, we have demonstrated a GPCR-independent novel mechanism whereby SP displays its pro-inflammatory effect by directly binding to the gp91phox and increasing superoxide production, which is critically linked with synergistic neurotoxic effects between SP and neurotoxins in PD.

NIEHS

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Visiting Fellow

Signal Transduction - General

p53 cooperates with MAP kinase and NF κ B signal transduction pathways to potentiate human immune/inflammatory response

The p53 tumor suppressor can regulate transcription of genes associated with a wide range of cellular functions including apoptosis, growth arrest, DNA repair, differentiation and glycolysis. Recently, we extended this list to include the Toll-like receptor (TLR) human innate immunity genes. TLRs are highly conserved integral membrane glycoproteins that recognize a variety of chemically distinct pathogen-associated molecular patterns (PAMPs). Upon stimulation, TLRs recruit adaptor molecules that lead to activation of NF κ B, interferon responsive factors (IRFs) and MAP kinases resulting in distinct patterns of gene expression essential to immune/inflammatory responses and elimination of pathogens. To determine the extent to which p53 dependent upregulation of the receptors could influence downstream response, we utilized a pair of isogenic cell lines with very different levels of p53: wild type p53 MCF7 breast adenocarcinoma cells or MCF7 cells stably transfected with p53 shRNA. Cells were pre-treated with Nutlin-3 to induce p53 and subsequent induction of TLR receptors (Nutlin-3 blocks the p53 inhibitor MDM2) and then exposed to TLR5 ligand flagellin. We found a dramatic (over 10-fold) p53-dependent amplification of cytokines IL-6 and IL-8 mRNA and protein production in response to flagellin. This change in response was accompanied by a specific increase in phosphorylation of p38 MAP kinase; p38 inhibitor SB203580 prevented the p53 dependent increase in IL-6 mRNA levels following flagellin exposure. On the contrary, IL-8 levels were not affected by p38 inhibition. To delineate the signaling pathway(s) linking p53 expression to p38 activation and enhanced cytokine production we performed gene expression analysis. The expression of over 200 genes was synergistically increased by a combination of flagellin and Nutlin-3 in a p53-dependent manner. Genes associated with Gene Ontology terms such as immune, defense and inflammatory response were significantly (p -value ≤ 0.0001) over represented. In addition, promoter analysis of the synergistic group demonstrated enrichment for NF κ B binding sites (p -value ≤ 0.0001). Interestingly, Nutlin-3 increased IL-6 and IL-8 expression also in response to TNF α that similarly utilize MAP kinase and NF κ B pathways demonstrating, therefore, that p53 can interact with these signaling pathways in a broad context. Our findings reveal a novel role for p53 to promote immune/inflammatory response.

NIEHS

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Stem Cells - General

Role of GLIS3 in the generation of pancreatic beta cells from ES and iPS cells

Diabetes mellitus is a major health concern presently affecting 10% of the US population. State-of-the-art insulin therapy does not prevent long-term complications from diabetes, therefore, development of beta cell replacement therapy might become an attractive alternative strategy. Recent studies have developed protocols to induce differentiation of (embryonic) stem cells into pancreatic endocrine cells, including β cells. However, many details of the mechanism controlling this differentiation process are still lacking. Our lab identified Krüppel-like zinc finger transcription factor Gli-similar 3 (Glis3) as a critical factor in the regulation of pancreatic β cells. Study of Glis3 knockout mice showed that these mice develop neonatal diabetes and lack the presence of beta cells. In addition, Glis3 plays an important role in the regulation of insulin transcription in matured β cells. Combined with the fact that mutations in Glis3 have been linked to type I/II diabetes in humans, we hypothesize that Glis3 may be critical for the development of pancreatic β cells. The goal of this study is to determine the role of Glis3 in the regulation of the differentiation of (embryonic) stem cells into pancreatic endocrine cells. We successfully induced differentiation of human adipose-derived stem cells (hASC), human embryonic stem cells (hESC), and induced pluripotent stem (iPS) cells into pancreatic endocrine cells and monitored the expression of various differentiation markers, including Pdx1, Ngn3, MafA, insulin, and glucagon, by real-time PCR and immunostaining. In these cell models, Glis3 mRNA expression was significantly induced during differentiation. We demonstrated that the expression of Glis3 was up-regulated as early as the definitive endoderm stage, but was greatly induced at the pancreatic progenitor cell stage. These observations suggested that Glis3 plays a role early in the differentiation of embryonic stem cells into pancreatic endocrine cells. To study the role of Glis3 in the differentiation of iPS cells into pancreatic endocrine cells, we generated iPS cells from wild type and Glis3 null mice. Our results suggest that Glis3 plays an important role in the differentiation of stem cells into β cells and expression of Glis3 might benefit cell replacement therapy against diabetes.

NIEHS

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Stem Cells - General

MicroRNA-mediated regulation of the BRG1 chromatin remodeling complex underlies the balance between pluripotency and differentiation in human embryonic stem cells

Embryonic stem (ES) cells hold great promise for regenerative medicine because of their unique characteristics of self-renewal and pluripotency. Balance between ES cell pluripotency and lineage commitment is maintained by gene expression networks that are largely dictated by chromatin structure. The BRG1 chromatin remodeling complex is required for mouse ES cell self-renewal and pluripotency and lineage specification during early murine development. Its role in human ES (hES) cells and early development, however, remains unclear. The complex is comprised of a central ATPase (BRG1) and multiple BRG1-associated factors (BAFs), which are assembled in a combinatorial fashion to dictate context-dependent functional specificity. Here we address the mechanisms by which this complex promotes pluripotency and early differentiation events in hES cells through changes in complex composition. We utilized in vitro culture and differentiation of hES cell lines to explore the regulation of BAFs in early human development. Through gain- and loss-of-function experiments we identified a

microRNA-mediated regulatory event critical for BAF regulation. The ES-cell specific miR-302 family directly represses BAF170 in hES cells. This repression is relieved upon differentiation and miR-302 inhibition. The importance of BAF170 repression for gene expression was explored through genome-wide microarray and RNA-seq studies. 352 genes were significantly affected at least 1.5 fold by BAF170 KD with 63% also misregulated upon miR-302 inhibition. Functional analysis revealed enrichment in Nodal signaling. qRT-PCR confirmed that miR-302 and BAF170 conversely regulate endodermal differentiation markers and targets of Nodal signaling, a pathway known to regulate both ES cell pluripotency and endodermal differentiation. Our data support a role for miR-302-mediated BAF170 repression in maintaining pluripotency through suppressing endodermal differentiation and suggest that relief of this inhibition is important for human endodermal lineage specification. This places the BRG1 complex at the center of cell fate decisions during early human development and provides mechanistic insight into the essential role of this complex in balancing stem cell pluripotency and differentiation. As the endodermal lineage gives rise to cells of the liver and pancreas, understanding these mechanisms will aid in the use of stem cell therapies for liver disease and diabetes.

NIEHS

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Vascular Disease and Biology

The deficiency of beta-arrestin2 attenuates abdominal aortic aneurysm formation in mice.

Abdominal aortic aneurysms (AAAs) are an increasing health concern, particularly in the aging male population. AAAs begin as a dilation of the abdominal aorta, which gradually expand over time due to inflammation and vascular remodeling. Currently, there are no pharmacological treatments for AAAs. A widely used mouse model to study AAAs involves chronic infusion of angiotensin II (AngII), and this model displays many characteristics of human AAAs. AngII mediates its effects primarily by activating the G-protein coupled angiotensin type 1 receptor (AT1). Recent studies have shown that the multifunctional scaffolding protein Beta-arrestin2 (Barr2) forms a complex with AT1 to initiate G-protein-independent signaling, which contributes to many pathophysiological conditions. To examine the role of Barr2 in AngII-induced AAA formation, Barr2-deficient (Barr2^{-/-}) and wild-type (Barr2^{+/+}) mice were infused with AngII (1000ng/kg/min, minipumps) for 28 days. AngII induced a 71% incidence of AAAs in Barr2^{+/+} mice, whereas only 15% of Barr2^{-/-} mice developed AAAs. AngII is a potent inducer of cyclooxygenase-2 (COX-2), an inflammatory enzyme that we have previously shown to be involved in AAA formation. Therefore, we hypothesized that increased COX-2 expression may be a mechanism by which Barr2 contributes to AAA pathology. AngII induced abundant COX-2 expression in the abdominal aortas of Barr2^{+/+} mice, whereas COX-2 expression was significantly attenuated in the aortas of Barr2^{-/-} mice, indicating that Barr2 is involved in the induction of COX-2 in response to AngII. An extensively studied pathway mediated by the Barr2-AT1 complex is ERK1/2 signaling, and ERK1/2 activation independently has been shown to be important in AAA development. Indeed, activated ERK1/2 was observed in the abdominal aortas of Barr2^{+/+} mice, but not in the abdominal aortas of Barr2^{-/-} mice. To determine if ERK1/2 activation was a mechanism for Barr2-AT1-induced COX-2 expression, Barr2^{+/+} mice were treated with the ERK1/2 inhibitor, CI1040 (100mg/kg) together with AngII and examined for COX-2 expression. ERK1/2 inhibition significantly attenuated AngII-induced COX-2 expression in the abdominal aortas of Barr2^{+/+} mice. Thus, Barr2 may contribute to AAA formation by activating the ERK1/2-COX-2 pathway, resulting in increased inflammation and vascular remodeling. These studies have important implications for the design of therapeutics that target Barr2-mediated signaling for the treatment of AAAs.